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Evidence towards the involvement of nitric oxide in drought tolerance of sugarcane

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Abbreviations: GSH, glutathione; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-nitrosoglutathione reductase; GSSG, oxidized glutathione; NH₄⁺, ammonium; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; PEG, polyethylene glycol; PPFD, photosynthetic photon flux density; RSNO, *S*-nitrosothiol; RWC, relative water content; WD, water deficit.

Abstract

Exogenous supplying of nitric oxide (NO) increases drought tolerance in sugarcane plants. However, little is known about the role of NO produced by plants under water deficit. The aim of this study was to test the hypothesis that drought-tolerance in sugarcane is associated with NO production and metabolism, with the more drought-tolerant genotype presenting higher NO accumulation in plant tissues. The sugarcane genotypes IACSP95-5000 (drought-tolerant) and IACSP97-7065 (drought-sensitive) were submitted to water deficit by adding polyethylene glycol (PEG-8000) in nutrient solution to reduce the osmotic potential to -0.4 MPa. For evaluating short-time responses to water deficit, leaf and root samples were taken after 24 h under water deficit. The drought-tolerant genotype presented higher root extracellular NO content, which was accompanied by higher root nitrate reductase (NR) activity as compared to the drought-sensitive genotype under water deficit. In addition, the drought-tolerant genotype had higher leaf intracellular NO content than the drought-sensitive one. IACSP95-5000 exhibited decreases in root *S*-nitrosogluthathione reductase (GSNOR) activity under water deficit, suggesting that *S*-nitrosogluthathione (GSNO) is less degraded and that the drought-tolerant genotype has a higher natural reservoir of NO than the drought-sensitive one. Those differences in intracellular and extracellular NO contents and enzymatic activities were associated with higher leaf hydration in the drought-tolerant genotype as compared to the sensitive one under water deficit.

Keywords: Nitrate reductase; *S*-nitrosogluthathione reductase; NO metabolism; genotype dependent.

1. Introduction

Despite evidence regarding the importance of nitric oxide (NO) in plant signaling, the mechanism responsible for NO synthesis is still controversial. It is now widely accepted that NO plays a key role in signaling among plant cells, however, it has been a challenge to determine the sources of NO in plants and there is considerable discussion of how exactly NO is formed in plant cells (Hancock, 2012; Salgado et al., 2013). In biological systems, NO can be formed both enzymatically and non-enzymatically. In mammals, the enzyme responsible for NO generation is NO synthase (NOS), with L-arginine being converted to citrulline, using NADPH as electron donor and O₂ as co-substrate and producing NO and water (Alderton et al., 2001). The existence of NOS remains questionable in plants. Although NO production is dependent on L-arginine and its production is sensitive to inhibitors of NOS (Moreau et al., 2010), a homologous gene for this protein has not been found in plants. While a recent extensive survey of higher plant genomes failed to uncover the presence of a NOS encoding region in any species (Jeandroz et al., 2016), Foresi et al. (2010) characterized the sequence, protein structure, phylogeny, biochemistry and NOS expression in green algae of the *Ostreococcus* genus, in which the amino acid sequence was 45% similar to human NOS.

The nitrate reductase (NR) enzyme is essential for nitrogen assimilation and also involved in NO production both *in vitro* (Rockel et al., 2002) and *in vivo* (Kaiser et al., 2002). In the first case, NR catalyzes the transfer of two electrons from NADPH to nitrate to produce nitrite. As secondary activity, NR also reduces nitrite to NO using NADPH, being NO synthesis dependent on nitrite content of plant tissues. The efficiency of this reaction for NO production is considered low and requires high concentrations of nitrite (Yamasaki and

Sakihama, 2000; Rockel et al., 2002). Modolo et al. (2005) have suggested that the primary role of NR for NO production is as a pathway to provide nitrite. Electrons required for the reduction of nitrite to NO can be provided by the mitochondrial respiratory chain (Planchet et al., 2005) or by the photosynthetic system (Jasid et al., 2006).

The NO bioavailability may be affected by glutathione (GSH), an antioxidant present at high intracellular concentrations. Spontaneous reaction of NO with the thiol grouping of GSH will form *S*-nitrosoglutathione (GSNO). The control of intracellular GSNO is partly regulated by degradation catalyzed by *S*-nitrosoglutathione reductase (GSNOR) (Frunghillo et al., 2014). The GSNOR catabolizes GSNO to oxidized glutathione (GSSG) and ammonium (NH_4^+), resulting in depletion of intracellular levels of GSNO and reduction of *S*-nitrosothiol (RSNO) formation by transnitrosation processes. In fact, GSNO has an important role in *S*-nitrosation and also represents a natural intracellular reservoir of NO (Ji et al., 1999; Liu et al., 2001).

Recent studies have shown that NO plays an important role in plants under stressful conditions, such as drought (Santisree et al., 2015; Farnese et al., 2016; Silveira et al., 2016). For instance, Arasimowicz-Jeloneka et al. (2009) found that *Cucumis sativus* subjected to mild water deficit enhanced NO synthesis in root cells, with an intense NO production in root elongation zone. Although several reports have shown increased NO production under drought (Filippou et al., 2011; Fan and Liu, 2012; Xiong et al., 2012; Cai et al., 2015), there is no information about how plant species/varieties differ in NO production and how this differential NO production is related to drought tolerance. The aim of this work was to test the hypothesis that drought-tolerance in sugarcane is associated with NO production and

metabolism, with the more drought tolerant genotype presenting higher NO accumulation in plant tissues.

2. Material and methods

2.1. Plant material and growth conditions

Two sugarcane genotypes (*Saccharum* spp.) developed by the Sugarcane Breeding Program of the Agronomic Institute (ProCana, IAC, Brazil) with differential biomass production and drought tolerance were studied: IACSP95-5000 is a drought-tolerant genotype (Marchiori, 2014), whereas IACSP97-7065 is sensitive to water deficit (Oliveira, 2012; Sales et al., 2013). Plants of both genotypes were obtained through vegetative propagation. Small stem segments (with one bud each) of mature plants were selected and planted on commercial substrate (Levington M2 Compost, Heerlen UK). After 50 days, plants with five to six leaves were transferred to modified Sarruge (1975) nutrient solution with 15 mmol L⁻¹ N (7% as NH₄⁺); 4.8 mmol L⁻¹ K; 5.0 mmol L⁻¹ Ca; 2.0 mmol L⁻¹ Mg; 1.0 mmol L⁻¹ P; 1.2 mmol L⁻¹ S; 28.0 µmol L⁻¹ B; 54.0 µmol L⁻¹ Fe; 5.5 µmol L⁻¹ Mn; 2.1 µmol L⁻¹ Zn; 1.1 µmol L⁻¹ Cu and 0.01 µmol L⁻¹ Mo. The pH of nutrient solution was kept between 5.5 and 6.0 and its electrical conductivity between 1.53 and 1.70 mS cm⁻¹ by weekly monitoring and corrected when necessary. Plants were grown in growth chamber, with a 12-h photoperiod, air temperature of 30/20°C (day/night), air relative humidity of 80% and the photosynthetic photon flux density (PPFD) about 700 µmol m⁻² s⁻¹.

2.2. Water deficit induced by polyethylene glycol (PEG)

Sugarcane plants growing in nutrient solution were submitted to water deficit (WD) by adding polyethylene glycol (PEG-8000, Fisher Scientific, Leicestershire, UK) to the solution. To prevent osmotic shock, PEG-8000 was added to the nutrient solution to cause a gradual decrease in its osmotic potential until -0.4 MPa. All evaluations were taken 24 hours after the solution reached the desired osmotic potential, being the short-term responses to water deficit evaluated. Leaf and root samples were collected, immediately immersed in liquid nitrogen and then stored at -80 °C for further enzymatic analyses.

2.3. Leaf relative water content (RWC)

The relative water content was calculated using the fresh (FW), turgid (TW) and dry (DW) weights of leaf discs according to Jamaux et al. (1997):
$$RWC = 100 \times [(FW - DW) / (TW - DW)].$$

2.4. Extracellular and intracellular NO detection

Diaminofluoresceins (DAFs) are markers used for detecting NO in tissues by fluorescence emission (Yao et al., 2004; Kojima et al., 1998). The chemical transformation of 4,5-diaminofluorescein compound (DAF-2) is based on the reactivity of aromatic diamines with NO in the presence of O₂. N-nitrosation of DAFs yields the highly green-fluorescent triazole form (DAF-2T) (Kojima et al., 1998). DAFs do not react directly with NO, but with their oxidized forms such as N₂O₃ (Mur et al., 2011). Among DAFs most used as NO

indicators, the plasma membrane-permeable compound DAF-2 diacetate (DAF-2DA) is prominent, being hydrolyzed by esterases to form NO-sensitive DAF-2 (Kojima et al., 1998). As DAF-2DA is able to enter the cell, this compound cannot be used to monitor NO extracellular content, unlike DAF-2 that is impermeable to plasma membrane.

Leaf and root samples (100 mg) were incubated in 10 mM Tris, 50 mM KCl, pH 7.2 buffer in 1 mL microcentrifuge tubes for 40 min, before the addition of 5 μ M 4,5-diaminofluorescein diacetate (DAF-2). The sample was placed into a quartz cuvette and fluorescence measured for 30 min (Suppl. Fig. S1) using a fluorescence spectrophotometer (F-2500, Hitachi Science & Technology, Berkshire, UK) with excitation and emission at 488 and 512 nm, respectively (Bright et al., 2009). For the negative control, samples were incubated in the absence of DAF-2. Data are shown as average value (n=3) for each treatment and they represent the fluorescence signal after 30 min, considering the negative control (data shown = sample – negative control).

Intracellular NO was visualized using the cell permeable NO-specific dye 4,5-diaminofluorescein-2 diacetate (DAF2-DA). Fresh leaf and root segments were incubated in MES-KCl buffer (10 mM MES, 50 mM KCl, 0.1 mM CaCl₂, pH 6.15), at room temperature for 15 min. Then, these segments were incubated in solution of 10 μ M DAF2-DA, mixing gently per 40 min in dark and at room temperature (Desikan et al., 2002; Bright et al., 2009). The samples were washed with buffer to remove the excess of DAF2-DA and placed onto a glass slide and covered with a glass slip before observing fluorescence using laser-scanning microscopy with excitation at 488 nm and emission at 515 nm (Nikon PCM 2000, Nikon, Kingston-upon-Thames, UK). Photos were taken with a 10x magnification, 15 s exposure

and 1x gain. Images were analyzed using ImageJ software (NIH, Bethesda, MD, USA) and data are presented as mean pixel intensities.

2.5. *S*-nitrosoglutathione reductase (GSNOR) activity

Leaf and root GSNO reductase activity was estimated spectrophotometrically as the rate of NADH oxidation in presence of GSNO as described previously (Frungillo et al., 2014). Briefly, 0.1 g of fresh tissue was grounded with liquid nitrogen, resuspended in 20 mM HEPES buffer, pH 8.0, 0.5 mM EDTA, 0.5 mM PMSF and proteinase inhibitors (50 mg mL⁻¹ TPCK and 50 mg mL⁻¹ TLCK) and centrifuged for 10 min at 10,000 g at 4 °C. The protein extract was then incubated with 20 mM HEPES buffer, pH 8.0, 350 µM NADH in the presence or not of 350 µM GSNO. GSNO reductase activity was estimated by subtracting the rate of NADH oxidation in the absence of GSNO from that in the presence of GSNO by using the NADH molar extinction coefficient (6.22 M⁻¹ cm⁻¹) and normalized by protein content.

2.6. Nitrate reductase (NR) activity

NR activity was estimated as the rate of NO₂⁻ production as described before (Frungillo et al., 2014). Protein extract was obtained from the macerate of 0.1 g of fresh tissue with liquid nitrogen in 20 mM HEPES, pH 8.0, 0.5 mM EDTA, 10 mM FAD, 5 mM Na₂MoO₄, 6 mM MgCl₂, 0.5 mM PMSF and proteinase inhibitors (50 mg mL⁻¹ TPCK and 50 mg mL⁻¹ TLCK). The reaction medium consisted of 1 mL of extraction buffer supplemented with 10

mM KNO₃ and 1 mM NADH. Nitrite production was determined by adding equal volumes of the reaction solution and 1% sulphanilamide, 0.02% N-(1-naphthyl) ethylenediamine dihydrochloride in 1.5 N HCl, and measurement of absorbance at 540 nm on a spectrophotometer. The values obtained were compared to those of a standard curve constructed using KNO₂ and normalized against protein content.

2.7. Protein content

The protein content was determined by the Coomassie-blue method (Bradford, 1976) using bovine serum albumin (BSA) as the standard. The readings were performed using the Fluostar Optima Microplate Reader (BMG Labtech, Ortenberg, Germany).

2.8. Data analysis

The experimental design was completely randomized and two causes of variation (factors) were analyzed: water availability and genotypes. Data were subjected to the analysis of variance (ANOVA) and mean values were compared by the Tukey test when significance was detected ($p < 0.05$). The results presented are the mean \pm SD and the number of replicates is stated in each figure legend.

3. Results

3.1. Leaf relative water content (RWC)

The water deficit induced a reduction in leaf RWC of both genotypes, with the drought-tolerant genotype being less affected as compared to the sensitive one (Fig. 1).

3.2. Extracellular and intracellular NO release

Leaves of the sensitive genotype showed a significant increase (+30.8%) in extracellular NO under water deficit, which was not found in the drought-tolerant genotype (Fig. 2A). In roots tissues, the extracellular NO production increased in both genotypes under water deficit as compared to well-hydrated plants. Remarkably, the drought-tolerant genotype exhibited the highest extracellular NO emission from roots under water deficit, being 46% higher than in roots of the sensitive genotype (Fig. 2B).

Intracellular NO content was monitored using the NO-sensitive probe DAF-2DA in a fluorimetric assay. Leaves of the drought-tolerant plants showed increase in fluorescence under water deficit when compared to well-hydrated condition (Fig. 3A,B). Non-significant changes in intracellular NO production were found in the sensitive genotype, regardless water availability. However, the drought-sensitive genotype presented lower values than the drought-tolerant genotype under low water availability (Fig. 3B). Both genotypes exhibited increases in intracellular NO content in roots under water deficit and no differences were noticed among the genotypes studied (Fig. 3C,D).

3.3. NO synthesis and degradation

Leaf NR activity was not affected by water deficit in both sugarcane genotypes (Fig. 4A). However, water deficit reduced root NR activity in both genotypes, with the drought-tolerant genotype presenting higher root NR activity than the drought-sensitive genotype under low water availability (Fig. 4B). Leaf GSNOR activity was not affected by water deficit and the drought-tolerant genotype presented higher GSNOR activity than the drought-sensitive one in both water conditions (Fig. 4C). Root GSNOR activity was reduced by water deficit only in the drought-tolerant genotype (Fig. 4D).

4. Discussion

The drought-tolerant genotype produced more NO extracellular in roots when compared to the drought-sensitive one (Fig. 2B). Such response may have a role in root formation, which would be expected under water deficit. In fact, NO is associated with the signaling cascades leading to root hair formation in *A. thaliana* (Lombardo et al., 2006, 2012) and with increases in root dry mass in sugarcane (Silveira et al., 2016). The main function of root hairs is to increase root surface and then improve the uptake of water and nutrients. In such context, increases in extracellular NO content could trigger root formation and improve water uptake in the drought-tolerant genotype, reducing the impact of low water availability on leaf water status (Fig. 1).

Images by confocal microscopy showed that leaves of the drought-tolerant genotype had also increased intracellular NO production under water deficit (Fig. 3A,B), giving additional evidence for an association between NO production and drought tolerance. It has been suggested that NO can diffuse rapidly through the cytoplasm and biomembranes, thus

affecting many biochemical functions simultaneously (Lamattina et al., 2003), although this has been questioned by Lancaster et al. (1997).

NO synthesis in plant cells is not yet fully understood, constituting one of the major challenges to studies investigating this signaling molecule. Nitrate reductase activity, a cytosolic enzyme essential for the assimilation of nitrogen, has been suggested to play a key role in NO production in plants (Horchani et al., 2011). In this study, the drought-tolerant genotype showed higher root NR activity than the drought-sensitive one under water deficit. However, root NR activity was lower in relation to NO emission in this same tissue (Figs. 2 to 4) and this can be explained by NO production through other enzymatic and non-enzymatic pathways (Hancock, 2012). The nitrite has been considered the main substrate for NO production and it can be reduced to NO by electrons provided by the electron transport chain in chloroplasts (Jasid et al., 2006) or by the mitochondrial chain (Planchet et al., 2005). Furthermore, polyamines (PAs) may induce NO biosynthesis in *Arabidopsis* seedlings, giving a new insight into PA-mediated signaling and NO as a potential mediator of PA actions (Tun et al., 2006). Interestingly, there were no short-term changes in leaf NR and GSNOR activities due to water deficit (Fig. 4A), revealing that increases in leaf NO content of the drought-tolerant genotype were induced by changes in other metabolic pathways, as discussed earlier.

NO degradation is as important as its synthesis in determining the final concentration of NO as a signaling molecule in plant cells. Herein, the drought-tolerant genotype exhibited decreases in root GSNOR activity under water deficit (Fig. 4D). As a consequence, it could be argued that GSNO is less degraded and this would improve the performance of plants under water deficit. In fact, GSNO regulates NO availability acting as a natural reservoir of

intracellular NO and acts particularly in *S*-nitrosation of thiol groups of proteins (Silveira et al., 2016). GSNOR can also modulate SNO levels in response to abiotic stresses, an important response for improving plant acclimation (Salgado et al., 2013). Accordingly, the drought-tolerant genotype exhibited higher leaf GSNOR activity than the sensitive one in both water regimes (Fig. 4A).

We found differential NO levels in sugarcane roots and leaves, with root showing higher intracellular and extracellular NO availability than leaves under water deficit (Figs. 2 and 3). In general, the root system perceives reductions in water availability and produces chemical signals that regulate water flow from roots to shoots (Tardieu, 1996). It has been shown that NO is one of these chemical signals and plays crucial role in stimulating the root system expansion and development (Silveira et al., 2016; Xu et al., 2017) and remodeling root cell wall (Moro et al., 2016).

NO influence on metabolic and physiological processes is due to its ability in interacting and modifying multiple targets within the plant cell (Lamattina et al., 2003), which turns the understanding of its effects on plants a hard task. In fact, the understanding of metabolic pathways controlling NO homeostasis in plants should be one of the major aims of NO research in the near future. Herein, we demonstrated that NO metabolism is more active in the drought-tolerant genotype than in drought-sensitive one, with the former presenting higher root extracellular NO content, higher root NR activity and lower root GSNOR activity as compared to the drought-sensitive genotype. In addition, the drought-tolerant genotype had higher leaf intracellular NO content than the drought-sensitive one. Further studies addressing long-term responses of plants to water deficit and how NO

modulates both physiological and morphological acclimation to varying water availability
should reveal more facets of this versatile signaling molecule in plants.

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Authors' contribution

NMS, LF, FCCM, JTH, IS, ECM and RVR designed the experiments. NMS and ES
performed the biochemical measurements. FCCM obtained the mini stalks taken from adult
plants. NMS and RVR wrote the manuscript and all authors contributed to data discussion
and edited the final version of the manuscript.

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Figure 1

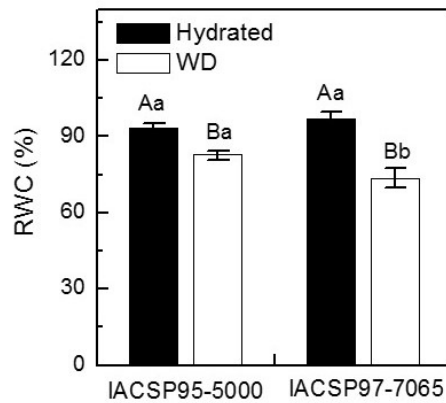


Fig. 1. Leaf relative water content (RWC) in sugarcane genotypes IACSP95-5000 (drought-tolerant) and IACSP97-7065 (drought-sensitive) under well-hydrated conditions (Hydrated) or water deficit (WD). The data represents the mean value of four replications \pm standard deviation. Different uppercase letters indicate statistical difference ($p < 0.05$) between water treatments, while different lowercase letters indicate statistical difference ($p < 0.05$) between genotypes.

Figure 2

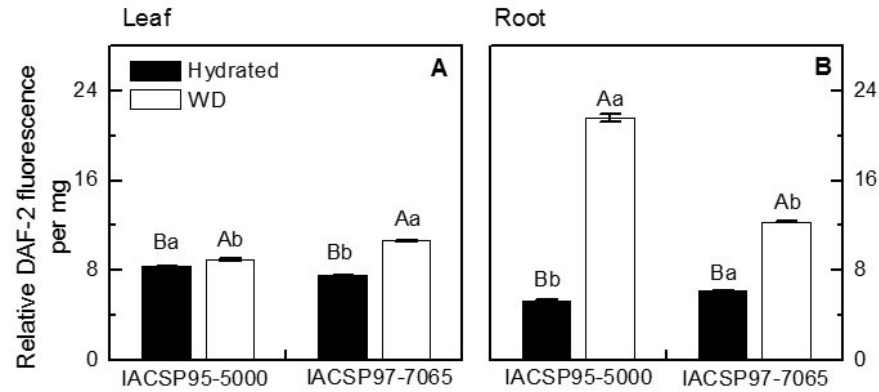


Fig. 2. Relative DAF-2 fluorescence demonstrating DAF-2-reactive compound-release (NO) in sugarcane genotypes IACSP95-5000 (drought-tolerant) and IACSP97-7065 (drought-sensitive) under well-hydrated conditions (Hydrated) or water deficit (WD) in leaves (A) and roots (B). The data represents the mean value of four replications \pm standard deviation. Measurements of relative fluorescence were taken after 30 min. Different uppercase letters indicate statistical difference ($p < 0.05$) between water treatments, while different lowercase letters indicate statistical difference ($p < 0.05$) between genotypes. Data were normalized by subtracting the values of the negative controls.

Figure 3

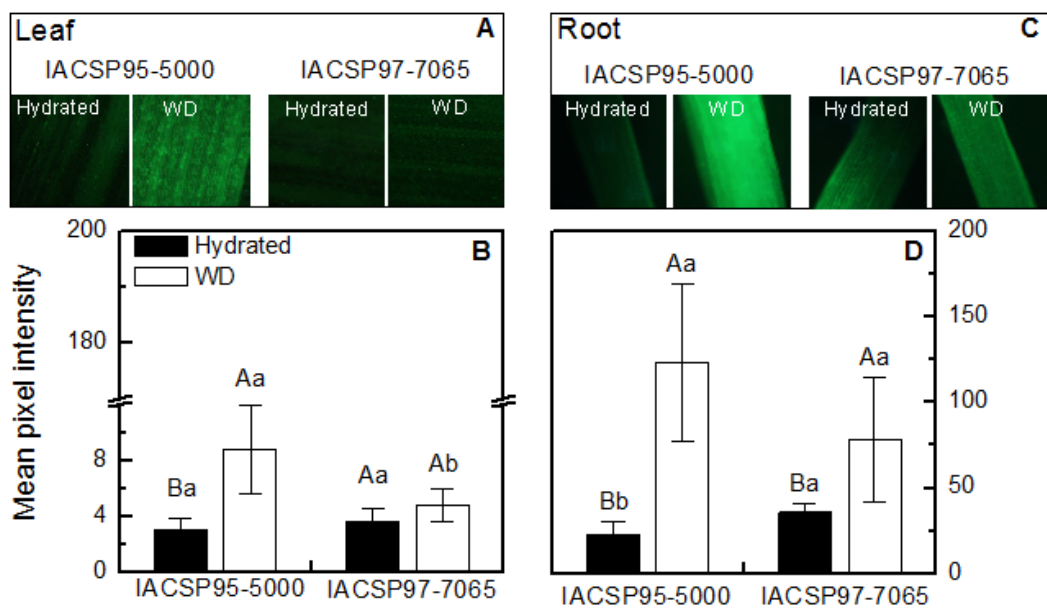


Fig. 3. Confocal microscopy images demonstrating intracellular NO synthesis in leaves (A) and roots (C) and mean pixel intensity by ImageJ in sugarcane genotypes IACSP95-5000 (drought-tolerant) and IACSP97-7065 (drought-sensitive) under well-hydrated conditions (Hydrated) or water deficit (WD) in leaves (B) and roots (D). The data represents the mean value of five replications \pm standard deviation. Different uppercase letters indicate statistical difference ($p < 0.05$) between water conditions, while different lowercase letters indicate statistical difference ($p < 0.05$) between genotypes. Data were normalized by subtracting the values of the negative control.

Figure 4

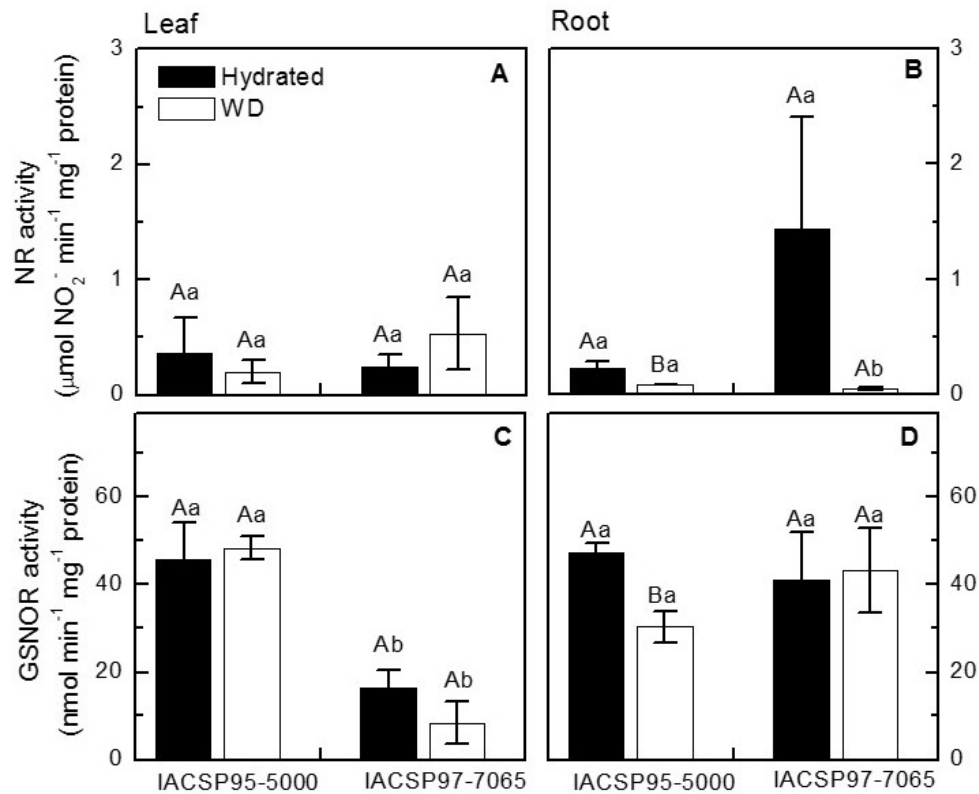


Fig. 4. Nitrate reductase activity (NR, in A,B) and *S*-nitrosoglutathione reductase activity (GSNOR, in C,D) in leaves (in A,C) and roots (in B,D) in sugarcane genotypes IACSP95-5000 (drought-tolerant) and IACSP97-7065 (drought-sensitive) under well-hydrated conditions (Hydrated) or water deficit (WD). The data represents the mean value of three replications \pm standard deviation. Different uppercase letters indicate statistical difference ($p < 0.05$) between water conditions, while different lowercase letters indicate statistical difference ($p < 0.05$) between genotypes.

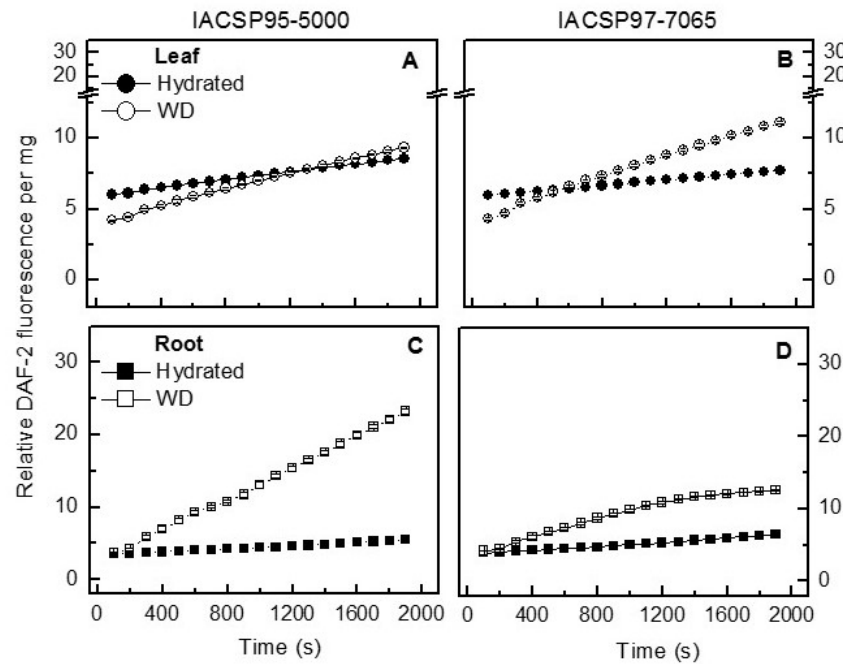


Fig S1. Relative DAF-2 fluorescence demonstrating DAF-2-reactive compound-release (NO) from leaves (in A,B) and roots (in C,D) of sugarcane genotypes IACSP95-5000 (drought-tolerant, in A,C) and IACSP97-7065 (drought-sensitive, in B,D) under well-hydrated conditions (Hydrated) or water deficit (WD). Measurements of relative fluorescence were taken every 100 s, for 30 min. The symbol represents the mean value of three replications \pm standard deviation. Data were normalized by subtracting the values of the negative controls.